

Evidence of ancient DNA reveals the first European lineage in Iron Age Central China

C. Z. Xie^{1,2}, C. X. Li², Y. Q. Cui^{1,2}, Q. C. Zhang¹,
Y. Q. Fu³, H. Zhu¹ and H. Zhou^{1,2,*}

¹*Ancient DNA Laboratory, Research Center for Chinese Frontier Archaeology, Jilin University, Changchun 130012, People's Republic of China*

²*College of Life Science, Jilin University, Changchun 130023, People's Republic of China*

³*College of Life Science, Jilin Agricultural University, Changchun 130118, People's Republic of China*

Various studies on ancient DNA have attempted to reconstruct population movement in Asia, with much interest focused on determining the arrival of European lineages in ancient East Asia. Here, we discuss our analysis of the mitochondrial DNA of human remains excavated from the Yu Hong tomb in Taiyuan, China, dated 1400 years ago. The burial style of this tomb is characteristic of Central Asia at that time. Our analysis shows that Yu Hong belonged to the haplogroup U5, one of the oldest western Eurasian-specific haplogroups, while his wife can be classified as haplogroup G, the type prevalent in East Asia. Our findings show that this man with European lineage arrived in Taiyuan approximately 1400 years ago, and most probably married a local woman. Haplogroup U5 was the first west Eurasian-specific lineage to be found in the central part of ancient China, and Taiyuan may be the easternmost location of the discovered remains of European lineage in ancient China.

Keywords: Yu Hong tomb; ancient DNA; mitochondrial DNA

1. INTRODUCTION

The Yu Hong tomb archaeological site (37°40'36" N, 112°25'36" E) is located in the northwestern Taiyuan basin in Shanxi Province (figure 1), China, and has been excavated since 1999. The burial style and multicolour relief have a Central Asian appearance, while the people pictured in the reliefs have obviously European morphological traits, such as straight noses and deep-set eyes. A well-preserved epitaph carved on the tombstone was found with the remains, with scenes depicting the life of Yu Hong (Zhang 2005) who died in AD 592, when he was 59 years old, and was buried in the same grave as his wife, who died later in AD 598. The ancestors of Yu Hong lived in ancient Yu country, which was located in the 'West Region,' which included Central Asia and Xinjiang at that time. His grandfather and father lived in ancient Xinjiang and were nobles of the Yu country. Yu Hong was a chieftain of the Central Asian people who had settled in China during the Sui Dynasty. However, anthropologists could not determine the race of these remains owing to the partial missing skulls. In order to discover their origin, it was decided to employ the technology of mitochondrial DNA analysis.

2. MATERIAL AND METHODS

(a) Sample preparation

One tooth and one femur sample were selected from Yu Hong's skeleton and from that of his wife. All samples were provided by the Cultural Relics and Archaeology Institute of Shanxi Province. The tooth and bone samples were processed independently. The tooth samples were immersed in 1 N hydrochloric acid for 3–5 min and washed with double

distilled water (ddH₂O), then with 100% alcohol. Then, all sides of the samples were exposed to ultraviolet (UV) light for 15 min. The samples were then pulverized by Freezer Mill 6750 (Metuchen, USA) after immersion in liquid nitrogen. On the other hand, 3–4 mm of the outer surface of the ancient bone fragment was removed, in order to eliminate possible surface contamination. After treatment with liquid nitrogen, the bone fragments were ground with a Planetary Mill Pulverisette 6 (Fritsch, Germany). All fine powders were stored at 4°C.

(b) Ancient DNA extraction

DNA was carefully extracted from the powdered bones and teeth samples using the GENECLEAN Kit for ancient DNA (BIO101, USA), according to its manual protocol.

(c) Uracil-N-glycosylase treatment

Ten microlitres of DNA extract from each of the samples were treated with 1 U of uracil-N-glycosylase (UNG) for 30 min at 37°C to excise uracil bases caused by the hydrolytic deamination of cytosines. UNG reduces sequence artefacts caused by this common form of post-mortem damage, which results in apparent G/C→A/T mutations and subsequent errors in sequence results (Gilbert *et al.* 2003). After this treatment, extracts were used to perform PCR amplification. The UNG treatment was useful for confirming doubtful sequences.

(d) PCR

The amplification of 364 bp (nucleotide positions; np 16035–16398) of the mtDNA HVR-I region was performed using four overlapping primer pairs (table 1; Xie *et al.* 2005). As a result of its relatively high evolutionary rate, it was both difficult and inadvisable to assign unique mtDNA haplogroups to specimens using control region DNA alone (Yao *et al.* 2002;

* Author for correspondence (zhouhui@mail.jlu.edu.cn).



Figure 1. Location of the Yu Hong cemetery.

Lalueza-Fox *et al.* 2004). Thus, five primers (table 1) were used to amplify mtDNA coding sequences for restriction fragment length polymorphism (RFLP) analysis.

The amplification reaction was set-up in a volume of 12.5 μ l, containing 1 \times PCR buffer (Promega, USA), 2.5 mM $MgCl_2$ solution (Promega, USA), 0.2 mM dNTP mix (Sangon, Shanghai, China), 2.5 U Taq DNA polymerase (Promega, USA), 0.2 μ M each primer, 2 mg ml⁻¹ BSA (Takara, Japan) and 1–5 μ l DNA from bone or teeth extracts. The cycle conditions using a Mastercycler gradient (Eppendorf, Hamburg, Germany) consisted of an initial denaturation at 94°C for 5 min, 36 cycles of 30 s at 94, 52–55 and 72°C, followed by a final extension at 72°C for 10 min.

(e) Sequencing

PCR products were visualized on 2% agarose gels stained with ethidium bromide and then purified using the QIAEX II Gel Extraction Kit (QIAGEN, German). Cycle sequencing was carried out with the ABI Prism BigDye Terminator Cycle Sequencing kit 3.1 (Applied Biosystems, USA) using 25 cycles at 92°C denaturing for 30 s, 15 s annealing at 55°C and elongation at 72°C for 2.5 min. The sequencing reaction was performed using the same primers as for PCR amplification. The sequences were collected by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA), according to the manufacturer's instructions.

(f) Quantification of amplifiable DNA

All samples were used to quantify the amplifiable DNA of three different fragment sizes: 138, 209 and 363 bp, by using the Gene Amp 5700 Sequence Detector (Applied Biosystems, USA). Real-time PCR amplification was performed in 25 μ l containing 1X SYBR GREEN PCR Master Mix (Applied Biosystems, USA), 0.5 μ M each primer, 2 mM BSA (Takara, Japan) and 5 μ l DNA extract.

(g) Cloning of PCR products

To minimize the risk of contamination, we cloned the mtDNA sequences from the bone samples of Yu Hong and his wife using the pGEM-T Easy Vector System I (Promega, USA), according to the manufacturer's instructions. Five white clones of each sample were selected for sequencing, using vector M13 primers.

(h) Authentication and prevention of contamination

In accordance with the suggested ancient DNA procedures (Cooper & Poinar 2000), the DNA extractions were performed in an isolated pre-PCR area that was exclusively

dedicated to ancient DNA, which contained positive air pressure, overnight UV-light (254 nm) exposure and frequent bench cleaning with DNase away (Molecular Bioproduct, San Diego, CA). Blank extraction and amplification controls were incorporated throughout the analysis. To help avoid laboratory contamination, sterile aliquoted reagents, sterile gloves, face masks, filter pipette tips and other standard precautions of ancient DNA studies were adopted.

Contamination during the excavation of skeletal remains is always a possibility and difficult to avoid. The best way to deal with this problem is by the simultaneous analysis of two independent samples from the same individual. For this reason, both bones and teeth of Yu Hong and his wife were analysed. In addition, the DNA of all laboratory personnel and archaeologists involved in the project was genetically typed and kept on record for a final comparison with the results.

Each sample was extracted three times, including one from the teeth samples and two from the bone samples, and then amplified at least three times to increase the reproducibility of results. After treatment with UNG, the post-mortem damaged portion was discarded. If more than one sequence had double peaks at the same position after treatment with UNG, it was treated as contamination, although the extraction and amplification blanks were always negative. Since the Taq misincorporation would not appear in the same position in independent amplifications of the same extract, the artefacts introduced by the Taq Polymerase could be identified.

3. RESULTS

(a) mtDNA sequence variation

We analysed mitochondrial DNA from Yu Hong and his wife's skeletons which were excavated from Yu Hong tomb in Taiyuan, Shanxi Province, dated AD 592. DNA was isolated from both the bones and teeth samples. Owing to the risk of contamination with modern DNA, we followed stringent and laborious protocols (Cooper & Poinar 2000).

The mtDNA HVR-I region analysis of each set of specimens resulted in the recovery of a 364-base pair (bp) fragment, 16035–16398 of the Cambridge Reference Sequence (CRS; Anderson *et al.* 1981), which was confirmed on each sequence of three independent extracts. The sequences recovered from the bone and tooth of each individual were identical. These sequences were compared to the CRS: there were a total of five variable np, all corresponding to transitions. Yu Hong had mutations at np 16243 T-to-C and 16270 C-to-T, versus his wife's at np 16223 C-to-T, 16260 C-to-T and 16362 T-to-C.

(b) Haplogroup distribution

Haplogroup classification of the ancient individuals was investigated according to the variations in both HVR-I sequence and coding region of mtDNA (Yao *et al.* 2002; Quintana-Murci *et al.* 2004). Nucleotide position 16270 was the characteristic transition of the U5 sub-haplogroup which belonged to macro-haplogroup R under macro-haplogroup N, while np 16223–16362 was the characteristic motif of haplogroup D or G, which were all in the macro-haplogroup M. Accordingly, the following sites were chosen for RFLP analysis: \pm 10397 *Alu* I indicated

Table 1. PCR primers used for DNA amplification in this study. (Number in primer name indicates position of 3' nucleotide.)

HVR-I/haplogroup	primer	sequence	length (bp)	annealing temperature (°C)
HVR-I	L16055	5'-GGAAGCAGATTTGGGTAC	128	52.5
	H16142	5'-ATGTACTACAGGTGGTCAAG		
HVR-I	L16131	5'-CACCATGAATATTGTACGGT	128	52
	H16218	5'-TGTGTGATAGTTGAGGGTTG		
HVR-I	L16185	5'-ACCCAATCCACATCAAAACC	142	55
	H16286	5'-TGTACTGTTAAGGGTGGGTAGG		
HVR-I	L16281	5'-CCTCACCCTAGGATACCAA	138	55
	H16379	5'-CAAGGGACCCCTATCTGAGG		
M/N	L10304	5'-ATGAGCCCTACAAACAAC	156	50
	H10423	5'-GAGTCGAAATCATTCTGTTT		
G	L4701	5'-CTATCCTCTTCAACAATATACTCT	182	53
	H4848	5'-ATGTGAGAAGAAGCAGGC		
D	L5159	5'-GCACCACGACCCTACTACTA	166	48
	H5287	5'-GGGATGATGAGGCTATTGT		
R	L12608	5'-ATCCCTGTAGCATTGTTCTG	151	52
	H12719	5'-GTTGGAATAGGTTGTTAGCG		
U	L12269	5'-TAACAACATGGCTTTCTCAACT	132	50
	H12357	5'-GAAGTCAGGGTTAGGGTGGT		

Table 2. Polymorphic restriction and haplogroup distribution of the ancient samples.

sample	10397 <i>Alu</i> I	5176 <i>Alu</i> I	4830 <i>Hae</i> II	12703 <i>Mbo</i> II	12308 <i>Hinf</i> I	haplogroup
Yu Hong	–			+	+	U5
Yu Hong's wife	+	+	+			G

macro-haplogroup M/N; +12703 *Mbo* II indicated macro-haplogroup R; +12308 *Hinf* I indicated haplogroup U; –5176 *Alu* I indicated haplogroup D; and +4830 *Hae* II indicated haplogroup G. The restriction enzyme results show that Yu Hong belonged to haplogroup U5, while his wife was categorized as a member of haplogroup G (table 2).

(c) Authentication of results

- (i) Experimental results showed an inverse correlation between the size of the amplified fragment and the efficiency of the amplification (table 3). This is consistent with one of the criteria used to determine authenticity in ancient DNA studies (Cooper & Poinar 2000; Alonso *et al.* 2004). In addition, the quantification results from the tooth samples show that starting copy numbers for amplification were more than 1000, confirming the sequencing results (Cooper & Poinar 2000).
- (ii) Since the quantity of DNA recovered from the bone samples was less than that from the tooth samples, cloning of PCR products of bone samples was performed. Five clones were selected for sequencing from each individual, and in these sequences, only a few positions were different from those in the direct sequencing. The consensus sequence of different clones, however, was consistent with the sequence obtained using the direct method. We determined the differences to have been caused by random Taq misincorporation. Post-mortem damage was ruled out because the extracts had been treated with UNG.

Table 3. Quantification results of three different fragment sizes.

fragment size (bp)	Yu Hong's bone	wife's bone	Yu Hong's tooth	wife's tooth
138	50–150 ^a	50–150	100–500	250–500
209	negative	negative	<30	<30
363	negative	negative	negative	negative

^a Quantification result showed the number of copies of DNA per microlitre extract, which was confirmed in three independent quantification PCRs.

- (iii) The sequences from the bone and tooth samples from each individual were identical, which strongly confirms the authenticity of the result.
- (iv) The DNA of all laboratory personnel and archaeologists involved in the project was genetically typed and found to be absent in all samples.

4. DISCUSSION

Yu Hong and his wife belong to different haplogroups. Yu Hong is included in haplogroup U5, the west Eurasian-specific haplogroup, while his wife belongs to haplogroup G, representative of East Asian populations.

Haplogroup U5 is the oldest European-specific haplogroup and its origin dates back to approximately 50 000 years. It most probably arose in the Near East and had spread into Europe in a very early expansion (Richards *et al.* 1996, 2000). Yu Hong's epitaph indicates that his ancestors lived in the ancient Yu country, which was located somewhere in the West Region, which

included Central Asia and Xinjiang in China at that time. However, Lin (2002) believed that it was located in Central Asia. The epitaph also tells us that Yu Hong's grandfather and father were nobles of Yu country and lived in ancient Xinjiang. Archaeologists believe that earlier ancestors may have arrived in ancient Xinjiang with the eastward expansion of Yu country (Zhang 2005).

In Central Asia, haplogroup U5 was especially concentrated in Tajikistan at approximately 4.5% frequency, and only three individuals from other Central Asian countries were of this type (Quintana-Murci *et al.* 2004). In Chinese Xinjiang, this haplogroup has been found in only 2 out of 53 samples from Kazaks in Kashen (Yao *et al.* 2004). From the analysis of the haplogroup U5 distribution, this haplogroup was concentrated mainly in Tajikistan and Kashen, both of which are located on the Pamirs. We theorize that Yu Hong came from this region and that the ancient Yu country may also have been located there.

The epitaph tells us that Yu Hong's grandfather and father still lived in Yu, and therefore he might have been the first person from his family who came to ancient China. The mtDNA lineage could not be passed on from the father, and we had no evidence that the women from his family, such as mother or sisters, had followed him. Thus, we could only confirm that this European matrilineal lineage had arrived here, but we did not know whether it had directly impacted the mtDNA lineages of human populations in this region. The migration of western peoples into East Asian populations has always interested biologists. As Comas wrote in his article 'The genetic influence of western peoples across Asia is obvious in Central Asia, but there is no evidence of its presence in the easternmost area since no traces are found in extant or ancient East Asian populations' (Comas *et al.* 2004). The analysis performed on the ancient sites of 2500-year-old Liangchun (Wang *et al.* 2000) and 2000-year-old Yixi (Oota *et al.* 1999), eastern China, concluded that there was a drastic shift from a European-like population (2500 years ago), through an intermediate population (2000 years ago), to the present day East Asian populations. However, re-examination of these data using a phylogeographic approach (Yao *et al.* 2003) clearly demonstrates no genetic link between these ancient populations and western Eurasians. On the other hand, our research shows that haplogroup U5 was the first west Eurasian-specific haplogroup to have been found in the central part of ancient China, and Taiyuan is the easternmost region where European lineage has been found until now in ancient China, despite the dubitable result of 2500-year-old Linzi (Wang *et al.* 2000).

Yu Hong wife's epitaph was badly preserved and gave us no information concerning her origin; consequently, we could deduce her origin only from an analysis of her DNA. The East Asian haplogroup G had never been found in the west, and only approximately 3–4% Central Asian mtDNAs fall into this haplogroup (Comas *et al.* 2004; Quintana-Murci *et al.* 2004). Other than the specific cases of G2a lineage (HVR-I motif 16223/16278/16227/16362), no other G sub-haplogroup seems to have expanded into Central Asia (Comas *et al.* 2004). Since the mutations of Yu Hong's wife did not match the characteristic motif of G2a and the haplogroup G was prevalent in East Asia, it appears that the matrilineal

ancestors of Yu Hong's wife may have no Central Asian origin and were instead local people.

In addition, the epitaph indicates that Yu Hong left the ancient Yu country as an emissary at the age of 13 (Zhang 2005). His epitaph also shows that he died 6 years before his wife. Since anthropologists believe that Yu Hong's wife was younger than her husband (Han 2005), she must have been no more than 5 years old at the time he left ancient Yu country. We believe that Yu Hong married in the North Qi of ancient China rather than Yu country, and his wife was most probably a local woman. Other scenarios are also possible.

Our findings indicate that a member of the oldest European-specific haplogroup, U5, arrived in Taiyuan approximately 1400 years ago. It is the first west Eurasian-specific haplogroup to have been found in the central part of ancient China, and Taiyuan may be the easternmost region where European lineage has been found until now in ancient China.

This research was supported by the National Natural Science Foundation of China (NSFC J0530184). The authors are grateful to Cultural Relics and Archaeology Institute of Shanxi Province, China, for providing the ancient samples, and to Valerie Clack, and Victor H. Mair, John Pinches and Songhe Wang for their revision on the manuscript.

REFERENCES

- Alonso, A. *et al.* 2004 Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Sci. Int.* **139**, 141–149. (doi:10.1016/j.forsciint.2003.10.008)
- Anderson, S. *et al.* 1981 Sequences and organization of the human mitochondrial genome. *Nature* **290**, 457–465. (doi:10.1038/290457a0)
- Comas, D., Plaza, S., Wells, R. S., Yuldaseva, N., Lao, O., Calafell, F. & Bertranpetit, J. 2004 Admixture, migrations, and dispersals in central Asia: evidence from maternal DNA lineages. *Eur. J. Hum. Genet.* **12**, 495–504. (doi:10.1038/sj.ejhg.5201160)
- Cooper, A. & Poinar, H. N. 2000 Ancient DNA: do it right or not at all. *Science* **289**, 1139. (doi:10.1126/science.289.5482.1139b)
- Gilbert, M. T. P., Willerslev, E., Hansen, A. J., Barnes, I., Rudbeck, L., Lynnerup, N. & Cooper, A. 2003 Distribution patterns of postmortem damage in human mitochondrial DNA. *Am. J. Hum. Genet.* **72**, 32–47. (doi:10.1086/345378)
- Han, K. X. 2005 Identification of the human remains of Yu Hong Tomb. In *Sui Dynasty Tomb of Yu Hong in Taiyuan*, pp. 209–234. Beijing, China: Cultural Relics Publishing House.
- Lalueza-Fox, C., Sampietro, M. L., Gilbert, M., Castri, L., Facchini, F., Pettener, D. & Bertranpetit, J. 2004 Unravelling migrations in the steppe: mitochondrial DNA sequences from ancient central Asians. *Proc. R. Soc. B* **271**, 941–947. (doi:10.1098/rspb.2004.2698)
- Lin, M. C. 2002 The research of Jihu history—several problem from the epitaph of the Yu Hong tomb. *Hist. Res. China* **1**, 71–84.
- Oota, H., Saitou, N., Matsushita, T. & Ueda, S. 1999 Molecular genetic analysis of remains of a 2000-year-old human population in China—and its relevance for the origin of the modern Japanese population. *Am. J. Hum. Genet.* **64**, 250–258. (doi:10.1086/302197)

- Quintana-Murci, L. *et al.* 2004 Where west meets east: the complex mtDNA landscape of the southwest and Central Asian corridor. *Am. J. Hum. Genet.* **74**, 827–845. (doi:10.1086/383236)
- Richards, M. *et al.* 1996 Paleolithic and Neolithic lineages in the European mitochondrial gene pool. *Am. J. Hum. Genet.* **59**, 185–203.
- Richards, M. *et al.* 2000 Tracing European founder lineages in the Near Eastern mtDNA pool. *Am. J. Hum. Genet.* **67**, 1251–1276.
- Wang, L., Oota, H., Saitou, N., Jin, F., Matsushita, T. & Ueda, S. 2000 Genetic structure of a 2500-year-old human population in China and its spatiotemporal changes. *Mol. Biol. Evol.* **17**, 1396–1400.
- Xie, C. Z., Liu, S. B., Cui, Y. Q., Zhu, H. & Zhou, H. 2005 Analysis of mitochondrial DNA from the ancient tombs of Chawuhu. *J. Jilin Univ. (Science Edition)* **43**, 538–540.
- Yao, Y. G., Kong, Q. P., Man, X. Y., Bandelt, H. J. & Zhang, Y. P. 2003 Reconstructing the evolutionary history of China: a caveat about inferences drawn from ancient DNA. *Mol. Biol. Evol.* **20**, 214–219. (doi:10.1093/molbev/msg026)
- Yao, Y. G., Kong, Q. P., Wang, C. Y., Zhu, C. L. & Zhang, Y. P. 2004 Different matrilineal contributions to genetic structure of ethnic groups in the Silk Road region in China. *Mol. Biol. Evol.* **21**, 2265–2280. (doi:10.1093/molbev/msh238)
- Yao, Y. G., Kong, Q. P., Bandelt, H. J., Kivisild, T. & Zhang, Y. P. 2002 Phylogeographic differentiation of mitochondrial DNA in Han Chinese. *Am. J. Hum. Genet.* **70**, 635–651. (doi:10.1086/338999)
- Zhang, Q. J. 2005 Research of the epitaph from the Yu Hong tomb. In *Sui dynasty tomb of Yu Hong in Taiyuan*, pp. 209–234. Beijing, China: Cultural Relics Publishing House.

NOTICE OF CORRECTION

The penultimate sentence of the Introduction is now correct.

9 May 2007